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The signal transduction protein phosphatidylinositol 3-kinase (PI3K) phosphorylates membrane constituent phosphatidylinositols, producing second messengers that link membrane bound receptor signals to cellular proliferation and survival. The dysregulation of the PI3K signal transduction pathway has been associated with tumorigenesis. The tumor suppressor PTEN/MMAC1 is a multispecific phosphatase that is a major negative regulator of PI3K, and PTEN/MMAC1 is frequently mutated in advanced cancers. We have produced four breast-targeted transgenic mouse models that express either the PTEN/MMAC1 wild-type or phosphatase-inactive proteins under the control of either the murine mammary tumor virus (MMTV) promoter or the whey acidic protein (WAP) promoter. These models are being characterized as part of our effort to understand the role of PTEN in breast tumorigenesis. In addition to these models, we have employed cell line model systems to further define the role PTEN/MMAC1 in PI3K signaling. We have determined that, dependent on the culture conditions, PTEN/MMAC1 expression can induce either apoptosis or cell cycle arrest in breast cancer cell lines and that loss of PTEN/MMAC1 is associated with an increase in the duration of ligand-induced signaling through the PI3K pathway. Our results are supportive of PTEN/MMAC1 being a breast tumor suppressor that functions by regulating the PI3K signaling pathway.

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Table of Contents

Introduction

The signal transduction protein phosphatidylinositol 3-kinase (PI3K) phosphorylates membrane constituent phosphatidylinositols, producing second messengers that link membrane bound receptor signals to cellular proliferation and survival (1). The tumor suppressor PTEN/MMAC1 is a multispecific phosphatase that is a major negative regulator of PI3K, and PTEN/MMAC1 is frequently mutated in advanced cancers (2). We proposed to develop transgenic mouse models that could be used to further the understanding of PTEN in mammary tumorigenesis. We have produced four breast-targeted transgenic mouse models that express either the PTEN/MMAC1 wild-type or phosphatase-inactive proteins under the control of either the murine mammary tumor virus (MMTV) promoter or the whey acidic protein (WAP) promoter. These models are currently being characterized. In addition to these transgenic animal models, we have employed breast cancer cell line model systems to further define the role PTEN/MMAC1 in regulation of the PI3K signaling pathway. This combined approach is being used to clarify the functional significance of tumor suppressor PTEN/MMAC1 in PI3K signaling pathway regulation as part of our effort to understand the role of PTEN in breast tumorigenesis.

Annual Summary

In our original proposal, our objectives included the development of transgenic mouse models that display mammary gland specific expression of the wild-type or phosphatase-inactive forms of PTEN/MMAC1. Our project timetable incorporated into the YEAR 1 statement of work included: 1) the development of breast-specific vector constructs for the production of PTEN/MMAC1 transgenic mice, 2) the actual development of the transgenic mouse lines, and 3) the characterization of the transgenic mice to determine the effect of wild-type or inactive PTEN/MMAC1 on the incidence of early breast tumorigenesis and mammary development. In addition, we have investigated the role of PTEN/MMAC1 in the regulation of the PI3K signal transduction pathway using a transient expression system in breast cancer cell lines.

To specifically target expression of the transgene to the mammary tissue, we have packaged the transgenes in constructs that lead to mammary specific expression. The murine mammary tumor virus promoter construct can potentially induce expression of the transgene throughout development, but has occasionally been associated with ectopic transgene expression. In contrast, the WAP promoter construct is reported to be more breast specific, but has a more limited developmental range of expression. In order to compensate for the potential shortcomings of either vector, we produced transgenic

mouse strains for both the wild-type and phosphatase inactive PTEN/MMAC1 transgenes using both constructs. We have screened candidate founder mice and have confirmed positive mice for each transgene for both promoters. We are currently in the process of evaluating the initial litters for copy number as well as the effect of transgene expression on mammary tissue development.

Concurrent with our transgenic studies, we have employed transient transfection approaches to express PTEN/MMAC1 in breast cancer cell lines to further our understanding of the role of PTEN/MMAC1 in regulation of PI3K signaling. Two breast cancer cell lines that have been reported to lack PTEN/MMAC1 protein are the MDA-MB-468 and BT-549 cell lines (3,4). We have found that in either of these cell lines, the epidermal growth factor-induced PI3K activity is markedly prolonged in comparison to other breast cancer cell lines that express endogenous PTEN/MMAC1. Strikingly, when MDA-MB-468 cells are transfected with PTEN/MMAC1 plasmid constructs, expression of PTEN/MMAC1 protein reduced the duration of ligand-induced PI3K activity to a level similar to that of cell lines expressing endogenous PTEN/MMAC1. However, when the phosphatase inactive PTEN/MMAC1 was expressed in MDA-MB-468 cells instead, the ligand-induced PI3K activity was once again prolonged. We observed a marked increase in expression of the cell cycle regulator $p27^{Kip1}$ in either MDA-MB-468 or BT-549 cells that had been infected with an adenovirus construct that contained PTEN/MMAC1, but

not empty virus, and this was associated with cell cycle arrest of MDA-MB-468 cells cultured in high serum conditions. When adenovirus-PTEN/MMAC1 infected BT-549 cells were cultured in low serum conditions instead, the cells underwent apoptosis. Altogether, our data support a role for PTEN/MMAC1 in cellular growth and survival through regulation of the PI3K signaling pathway.

We have also conducted studies on mechanisms of the PI3K pathway regulation not directly associated with PTEN/MMAC1. In recently published work, we demonstrated that tyrosine phosphatase SHP-1 could physically associated with and dephosphorylate the PI3K regulatory subunit p85, and that this was associated with a decrease in phosphotyrosine-associated PI3K activity. This suggested to us that tyrosine phosphorylation of p85 was important in the regulation of PI3K activity or function. We conducted mutational studies in which the primary site of phosphorylation of p85 (Y688) by protein tyrosine kinase Lck was mutated to aspartate in order to mimic a phosphorylated tyrosine residue, or to alanine to produce an non-phosphorylatable p85. We observed the aspartate mutant p85 (phosphorylated mutant) expression to be associated with significantly higher PI3K activity than either wild-type or alanine mutant p85 (non-phosphorylated mutant), suggesting that p85 phosphorylation is important in the regulation of PI3K activity. Further, this activity difference between phosphorylated and non-phosphorylated PI3K was translated into significantly altered activity in

downstream protein NFκB. Finally, wild-type p85 expression was associated with a marked reduction in cell survival as compared to that of cells that express the aspartate mutant p85 (phosphorylated mutant). Altogether, the data from this branch of our studies suggest that tyrosine phosphorylation of PI3K is also important in the regulation of PI3K activity.

In summation, our work the past year has yielded the following **key research accomplishments**:

-successful production of breast-specific expression of tumor suppressor transgene
in a mouse model.

-we have published work describing the biochemical and functional effect of PTEN/MMAC1 expression in breast cancer cells.

-we have published work describing the relationship between PI3K and the protein tyrosine phosphatase SHP-1 and the regulatory role of SHP-1 in PI3K signaling.

-we have submitted for publication our data from which we describe the role of tyrosine phosphorylation in the regulation of PI3K.

Conclusions: We conclude that when transiently expressed in breast cancer cells lacking endogenous protein, PTEN/MMAC1 can regulate cell cycle progression or survival

depending on the culture conditions. Further, PI3K activity is, in part regulated by the phosphorylation state of the p85 subunit.

Reportable Outcomes

1) We have established two sets of transgenic mouse models with mammary specific expression of wild-type and phosphatase-inactive PTEN/MMAC1. Each set is under the control of different mammary specific promoters to ensure that we have working models with the desired specific expression pattern.

2) We have experimental evidence to support a role for PTEN/MMAC1 in the regulation of cell survival and growth. Further, our data suggests that loss of PTEN/MMAC1 activity in some breast cancer cell lines is associated with prolonged ligand-induced PI3K signaling, and that this is associated with increased cell survival.

3) Recent data from our lab is supportive of a role for tyrosine phosphorylation in the regulation of PI3K activity and function. We have produced mutant PI3K constucts which our data suggests mimic tyrosine phosphorylated and non-phosphorylatable p85, and phosphorylation of p85 is associated with significantly enhanced PI3K signaling as compared to wild-type.

4) Publications:

5) 1) Lu, Y., Lin, Y., LaPushin, R., Cuevas, B., et al. (1999) The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene. 18. 7034-7045.

6) 2) Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K.A., and Mills, G.B. 1999 SHP-1 regulates lck-induced phosphatidylinositol 3-kinase phosphorylation and activity. J Biol Chem. 1999.274(39).27583-9.

7) 3) Cuevas, B., Lu., Y., LaPushin, R., Mills, G.B. (2000) Tyrosine phosphorylation of p85 relieves its inhibitory activity on PI3K.Submitted.

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The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells

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The PTEN/MMAC1/TEP (PTEN) tumor suppressor gene at 10q23.3 is mutated in multiple types of sporadic tumors including breast cancers and also in the germline of patients with the Cowden's breast cancer predisposition syndrome. The PTEN gene encodes a multifunctional phosphatase capable of dephosphorylating the same sites in membrane phosphatidylinositols phosphorylated by phosphatidylinositol 3′-kinase (PI3K). We demonstrate herein that loss of PTEN function in breast cancer cells results in an increase in basal levels of phosphorylation of multiple components of the P13K signaling cascade as well as an increase in duration of ligand-induced signaling through the P13K cascade. These alterations are reversed by wild-type but not phosphatase inactive PTEN. In the presence of high concentrations of serum, enforced expression of PTEN induces a predominant G1 arrest consistent with the capacity of PTEN to evoke increases in the expression of the p27Kip1 cyclin dependent kinase inhibitor. In the presence of low concentrations of serum, enforced PTEN expression results in a marked increase in cellular apoptosis, a finding which is consistent with the capacity of PTEN to alter the phosphorylation, and presumably function, of the AKT, BAD, p70S6 kinase and GSK3α apoptosis regulators. Under anchorage-independent conditions, PTEN also induces anoikis, a form of apoptosis that occurs when cells are dissociated from the extracellular matrix, which is enhanced in conjunction with low serum culture conditions. Together, these data suggest that PTEN effects on the PI3K signaling cascade are influenced by the cell stimulatory context, and that depending on the exposure to growth factors and other exogenous stimuli such as integrin ligation, PTEN can induce cell cycle arrest, apoptosis or anoikis in breast cancer cells.

Keywords: PTEN/MMAC1; PI3-K (phosphatidylinositol 3-kinase); breast cancer; apoptosis; anoikis; cell cycle arrest

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Introduction

PTEN (a.k.a. MMAC1 and TEP) is a recently identified tumor suppressor gene located on human chromosome 10q23.3 (Li and Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997). Deletions and mutations in PTEN occur frequently in advanced cancers including breast cancer, glioblastoma multiforme, endometrial carcinoma, malignant melanoma, bladder carcinoma, small cell lung cancer, and endometrioid ovarian cancer (Parsons, 1998; Teng *et al.*, 1997). Germ-line mutations of PTEN are the cause of Cowden's and Bannayan-Zonana breast cancer predisposition syndromes, conditions in which 80 per cent of the associated tumors that arise are breast cancers (Liaw *et al.*, 1997; Marsh *et al.*, 1997). PTEN is also required for embryogenesis, but embryonic stem cells lacking functional PTEN have greater tumorigenic capability and heterozygous PTEN 'knockout' mice manifest the bowel hamartomas characteristic of Cowden's syndrome as well as an increased propensity for tumor development (Di Cristofano *et al.*, 1998; Suzuki *et al.*, 1998; Podsypanina *et al.*, 1999)

PTEN encodes a 403-amino-acid phosphatase capable of dephosphorylating phosphorylated serine, threonine and tyrosine residues (Myers *et al.*, 1997). PTEN interacts with the focal adhesion kinase (FAK) and, by virtue of its protein tyrosine phosphatase activity, PTEN reduces FAK phosphorylation and activity so as to alter cell adhesion, spreading and migration (Tamura *et al.*, 1998, 1999). Importantly, PTEN also has the ability to dephosphorylate position D3 of phosphatidylinositol (3,4,5) trisphosphate, the latter of which represents a direct product of phosphatidylinositol 3-kinase (PI3K) activity (Maehama and Dixon, 1998). Strikingly, a number of germline and sporadic mutations in PTEN, such as the substitution of glutamic acid for the glycine residue at position 129 (G129E), ablate the ability of PTEN to dephosphorylate phosphatidylinositol (3,4,5) trisphosphate, but leave PTEN protein phosphatase activity intact (Furnari *et al.*, 1998; Myers *et al.*, 1997, 1998). The association of these mutations with cancer, combined with the capacity of the G129E mutated form of PTEN to dephosphorylate FAK and regulate cell spreading in a fashion indistinguishable from wild-type PTEN (Tamura *et al.*, 1998), indicates that dephosphorylation of phosphatidylinositol (3,4,5) trisphosphate represents a function of PTEN critical to this protein's, ability to act as a tumor suppressor.

Enforced expression of PTEN has been reported to result in decreased cell proliferation and decreased tumorigenicity (Cheney *et al.*, 1998, 1999; Furnari *et al.*, 1998; Li and Sun, 1998; Tamura *et al.*, 1999; Tian *et al.*, 1999), an observation attributed to the ability of PTEN to induce cell cycle arrest, apoptosis or anoikis, a form of apoptosis that occurs when cells are dissociated from their extracellular matrix (Davies *et al.*, 1998, 1999; Frisch and Ruoslahti, 1997; Furnari *et al.*, 1998; Li and Sun, 1998; Sun *et al.*, 1999; Ramaswamy *et al.*, 1999; Li *et al.*, 1998; Stambolic *et al.*, 1998). However, a consensus has not been reached on whether the capacity of PTEN to modulate these processes is determined by cell lineage or culture conditions. Similarly, the biochemical mechanisms whereby PTEN influences cell behavior are not well understood. However, enforced expression of PTEN has been linked to the decreased phosphorylation or activity of AKT, BAD and 4E-BP1 proteins, which act downstream of PI3K in various signaling cascades (Datta *et al.*, 1997; Davies *et al.*, 1998, 1999; Delcommenne *et al.*, 1998; Haas-Kogan *et al.*, 1998; Pap and Cooper, 1998; Wu *et al.*, 1998; Tian *et al.*, 1999; Ramaswamy *et al.*, 1999) and increased expression of p27, which is also downstream of PI3K (Cheney *et al.*, 1999; Sun *et al.*, 1999).

In view of the central role for PTEN in the Cowden's breast cancer predisposition syndrome and its frequent mutation in breast cancers, we have investigated the ability of PTEN to regulate signaling and biological functions of breast cancer cells. We report, herein, that PTEN downregulates PI3K-dependent phosphorylation and the activation of diverse downstream targets of the PI3K signaling cascade. This inhibitory effect of PTEN requires an intact phosphatase domain. When overexpressed in breast cancer cells, PTEN can inhibit cell cycle progression, induce apoptosis and accelerate anoikis, but these properties of PTEN are modulated by the activity of growth factors present in serum as well as concomitant anchorage-dependent cell activation, for example through ligation of integrin receptors. These data also indicate the effects of PTEN on cell cycle progression and cell death to be attributable, at least in part, to its ability to alter phosphorylation of the AKT, p70S6 kinase, BAD and GSK3 apoptotic mediators and expression of the p27Kip1 cell cycle regulator.

Results

PTEN regulates duration of signaling through PI3K

PTEN has the potential to function as an antagonist of PI3K-dependent signaling in breast cancer cells (Li *et al.*, 1998). To address this possibility, PI3K-dependent signaling was investigated in breast cancer cell lines with and without PTEN mutations. As shown in Figure 1, PTEN protein was not detected in two breast cancer cell lines, MDA-MB-468 and BT-549, which carry deletion and frame shift mutations at codons 70 and 274, respectively (Li *et al.*, 1997, 1998). By contrast, the SKBr3, MCF7 and MDA-MB-231 breast cancer cell lines express similar amounts of PTEN as do normal breast epithelium (NB018) and the immortalized normal breast epithelial cell lines, MCF10A and MCF10F compatible with a lack of PTEN sequence abnormality in SKBr3, MCF7 and MDA-MB-231 (Li *et al.*, 1998). As shown in Figure 2A, breast cancer cells expressing mutant PTEN (MDA-MB-468 and BT-549) display higher levels of basal (time 0) AKT phosphorylation, indicative of increased PI3K signaling, than do cells expressing wild-type PTEN (SKBr3 and MDA-MB-231). EGF induced marked increases in AKT phosphorylation in the MDA-MB-468, SKBr3 and MDA-MB-231 cells (Figure 2A). In BT-549 cells, EGF induced modest, if any, effects on AKT phosphorylation, an observation which may reflect the high basal levels of AKT phosphorylation in these cells (Figure 2A). Strikingly, the duration of EGF-induced AKT phosphorylation was markedly increased in cells with mutant PTEN (MDA-MB-468), with levels of AKT phosphorylation remaining increased at 4 h after activation. By contrast, AKT phosphorylation declined in SKBr3 and MDA-MB-231 cells by 1 h after activation and returned to basal levels by 4 h.

To confirm that the high basal and prolonged duration of AKT phosphorylation in MDA-MB-468 cells was due to mutations in PTEN and not due to overexpression of the EGFR (Bates *et al.*, 1990), wild-type PTEN was reintroduced into these cells and its effects on AKT phosphorylation was examined. As indicated in Figure 2B, introduction of wild-type PTEN resulted in a marked decrease in basal phosphorylation and in the duration of ligand-induced phosphorylation of co-transfected AKT. The effect of PTEN on AKT phosphorylation persisted for at least 48 h in the presence or absence of serum (not presented). The effect of PTEN on AKT phosphorylation required the phosphatase activity of PTEN, as no differences were detected between the effects of a vector control and those observed using a mutated form of PTEN (PTEN C124S), which ablates both protein and lipid phosphatase activity (Figure 2B). PTEN lipid phosphatase activity was also critical to the kinetics of AKT phosphorylation as AKT phosphorylation was not altered by a mutated form of PTEN (PTEN G129E) (Figure 2B) in which phosphatidyinositol 3′ phosphatase activity of PTEN is lost, but protein phosphatase activity remains intact (Funari *et al.*, 1998; Myers *et al.*, 1998) (Figure 2B).

PTEN regulates phosphorylation of multiple components of the PI3K signaling cascade

As indicated by the immunoblotting data shown in Figure 3, introduction of wild-type PTEN, but not catalytically inactive PTEN (C124S), into BT-549 and MDA-MB-468 cells, resulted in marked decreases in the phosphorylation of multiple downstream components of

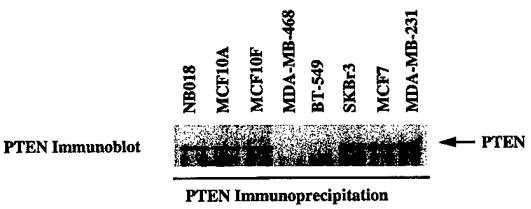


Figure 1 Expression of PTEN in breast cell lines. Endogenous PTEN was immunoprecipitated from 500 μg of cellular protein using goat anti-PTEN, separated by 8% SDS–PAGE and immunoblotted with rabbit anti-PTEN

the PI3K signaling cascade, including AKT (serine 473), BAD (serine 112 and serine 136), GSK3α (serine 21) and p70S6 kinase (serine 411, threonine 421 and serine 424). Thus PTEN appears to modulate a number of phosphorylation events known to be relevant to the functions of these PI3K pathway components (Coffer *et al.*, 1998; Pullen and Thomas, 1997). With each of these effectors, PTEN was found to be more effective at decreasing basal than at decreasing fetal bovine serum or EGF-stimulated levels of phosphorylation. Similar effects on basal phosphorylation were observed in the context of both plasmid and adenoviral-mediated PTEN expression and also through the use of the relatively specific PI3K inhibitor, wortmannin. By contrast, ligand-induced phosphorylation was more efficiently reversed by wortmannin treatment than by PTEN overexpression (Figure 3). Both rapamycin and wortmannin also decreased p70S6 kinase phosphorylation, a result consistent with data placing the p70S6 kinase downstream of PI3K and the rapamycin-sensitive FRAP/TOR PI3K superfamily member (Chou and Blenis, 1995). Strikingly, overexpression of PTEN in breast cancer cells containing wild-type PTEN (SKBr3 and MCF-7) did not alter either basal or induced AKT phosphorylation (Figure 3D). This result was in marked contrast to that detected in wortmannin treated cells, in which phosphorylation was effectively suppressed independent of the presence or absence of functional PTEN (Figure 3). These data suggest that PTEN levels are not limiting in these cell lines and raise the possibility that induced PTEN overexpression may markedly influence tumor cells while having limited effects on normal cells. Taken together, these results indicate that PTEN acts to suppress basal activity of the PI3K pathway but has a much less significant effect on ligand-induced PI3K signaling. The data also suggest

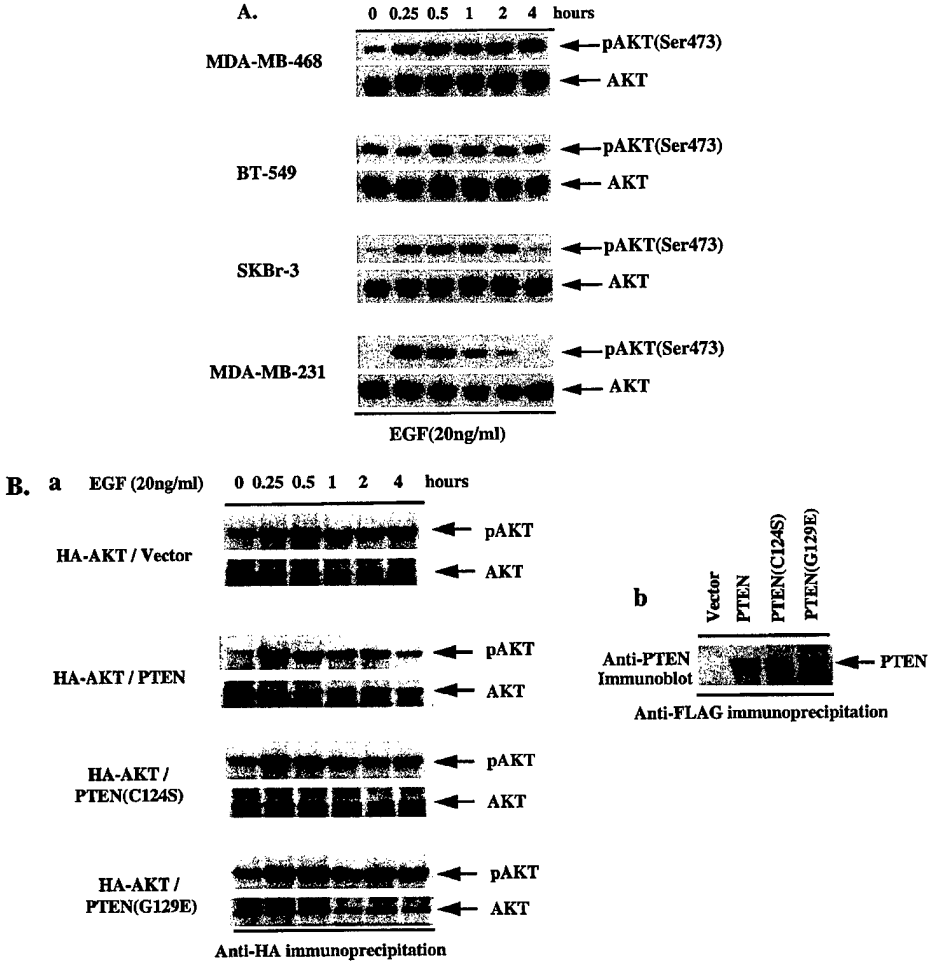


Figure 2 PTEN regulates duration of EGF-induced AKT activation. Cells were serum starved overnight prior to stimulation with EGF (20 ng/ml). (**A**) 12 μg of cellular protein was separated by 8% SDS–PAGE and immunoblotted with anti-phospho-AKT and reprobed with anti-AKT to confirm equal loading. (**B**) MDA-MB-468 cells were cotransfected with HA-AKT and empty vector, wild-type FLAG-PTEN, protein and lipid phosphate inactive mutant of PTEN (C124S) or lipid phosphatase inactive but protein phosphatase competent PTEN (G129E). Cell lysates were immunoprecipitated with anti-HA, separated by 8% SDS–PAGE and immunoblotted with anti-phospho-AKT (Ser473) and reprobed with anti-AKT to confirm equal loading and equivalent transfections. PTEN expression was assessed by immunoprecipitation with anti-FLAG (M2) and immunoblotting with rabbit anti-PTEN

that PTEN activity is, in the same manner, suppressed in conjunction with ligand evoked PI3K signaling.

PTEN increases expression of p27Kip1

As indicated in Figure 4A,B, expression of PTEN in BT-549 or MDA-MB-468 was also associated with increased expression of the p27Kip1 cyclin dependent kinase inhibitor. In contrast to the data shown in Figure 3, the effects of PTEN on p27Kip1 are most clearly manifested in the presence of ligand, possibly reflecting the fact that p27Kip1 expression levels are lower in stimulated than unstimulated cells (Figure 4). In contrast, under the same conditions, PTEN only modestly downregulates expression of the p53-dependent cyclin dependent kinase inhibitor, p21Cip1/Waf1 (data not shown).

PTEN inhibits cell proliferation

The data concerning PTEN effects on p27Kip1 expression and on phosphorylation of AKT, BAD, GSK3α and p70S6K raised the possibility that PTEN expression in BT-549 or MDA-MB-468 might alter cell cycle progression as well as cell sensitivity to apoptosis. As indicated in Figure 5, expression of PTEN in BT-549 or MDA-MB-468 cells resulted in lower proliferation rates (as indicated by MTT dye conversion) than those detected in mock infected or empty virus (DE1) infected cells in the presence of high (10%) or low (0.5%) fetal bovine serum. This effect of PTEN on cell proliferation was similar to that induced by LY294002, a PI3K inhibitor, utilized in these studies due to its decreased toxicity compared to wortmannin (the LY294002 concentrations used are equally as effective as wortmannin at inhibiting PI3K, data not presented). As is consistent with the lack of PTEN effect on AKT phosphorylation in cells containing intact PTEN (Figure 3), expression of PTEN did not decrease the proliferation of T47D breast cancer cells expressing wild-type PTEN (not presented).

PTEN alters cell cycle progression, apoptosis and anoikis dependent on culture conditions

The association of PTEN expression with reduced proliferation of MDA-MB-468 and BT-549 cells (Figure 5) may reflect cell cycle arrest and/or an increase in the fraction of cells undergoing apoptotic cell death. Under anchorage dependent conditions, in which integrins may be ligated by extracellular matrix components (Frisch and Ruoslahti, 1997) and in the presence of high concentrations of serum, expression of PTEN was found to be associated with increases in the percentage of MDA-MB-468 cells (Figure 6 and Table 1) and BT-549 cells (not presented) in the G1 phase of the cell cycle, a result indicative of cell cycle arrest. This observation is compatible with the detection of increased P27Kip1 expression in these cells following introduction of PTEN (Figure 4). PTEN did not induce apoptosis under anchorage-dependent conditions in the presence of high serum (10%), as indicated by the results of propidium iodide staining (Figure 6), a TdT-based apoptosis assay (Figure 7A) and by exposure of the Apo 2.7 apoptosis-related mitochondrial protein (Table 2. Koester *et al*., 1997; Pepper *et al*., 1998). These findings are consistent with the data revealing the effects of serum on phosphorylation of the AKT, p70S6 kinase, GSK3α and BAD apoptotic regulators to be only partially antagonized by introduction of PTEN (Figure 3); and the effects of serum on the RAS/MAPK pathway, which can prevent apoptosis (Xia *et al*., 1995; Downward, 1998; Werner and Le Roith, 1997), to be completely resistant to the effects of PTEN (not presented, Davies *et al*., 1998).

As illustrated by the data shown in Figure 3, the effects of PTEN on signaling through the PI3K pathway are most clearly manifest in the absence of serum. Strikingly, when BT-549 and MDA-MB-468 cells were cultured under anchorage dependent conditions in the presence of limiting concentrations of serum (0.5%), the cells underwent a significant degree of apoptosis as assessed by DNA fragmentation and exposure of the Apo 2.7 antigen (Figure 7A, Table 2). The PI3K inhibitors, LY294002 and wortmannin (Figure 7A), also induced increased apoptosis under these conditions, an observation consistent with the capacity of PTEN expression to decrease signaling through the PI3K pathway.

The PI3K pathway has been implicated in the prevention of anoikis, a form of apoptosis initiated by disruption of interactions between transmembrane integrins and ligands in the extracellular matrix (Frisch and Ruoslahti, 1997; Howe *et al*., 1998). BT-549 and MDA-MB-468 cells were cultured under anoikis conditions (inability to adhere to plastic due to rocking), expression of PTEN induced an increased rate of anoikis in the presence of high concentrations of serum as indicated by the presence of free DNA ends (Figure 7B) and by PARP cleavage (not presented). The effect of PTEN on anoikis, as on apoptosis, was most marked, however, when cells were cultured in the presence of low concentrations of serum (0.5%). Thus, PTEN modulates cell cycle progression, apoptosis and anoikis and its effects on those facets of cell behavior depend upon cell culture conditions, factors present in serum as well as integrin ligation.

Breast cancer cells with mutant PTEN are more sensitive to the growth inhibitory effects of PI3K inhibitors

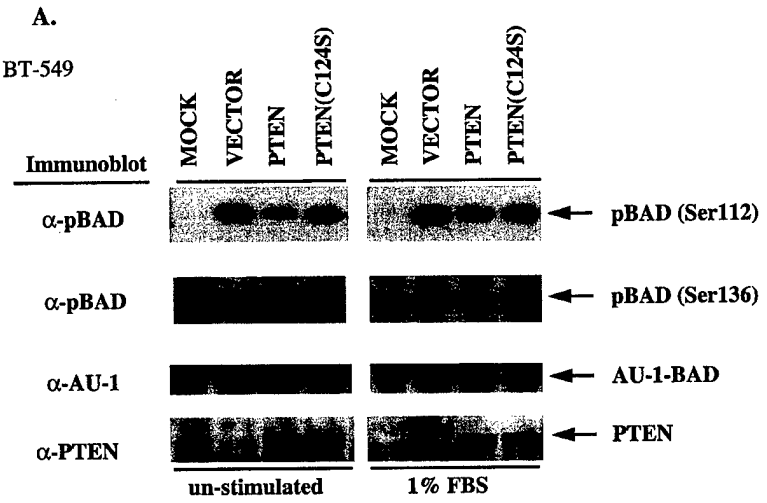
As noted above, the effects of PTEN on signaling and cell proliferation are most clearly manifest in cells that lack functional PTEN. In addition, inhibitors of PI3K exert similar effects on the PI3K signaling cascade as does enforced PTEN expression in cells lacking functional PTEN (Figure 3). As indicated in Figures 5 and 7, the specific PI3K inhibitor LY294002 decreases proliferation and induced apoptosis and anoikis in BT-549 and MDA-MB-468 cells. In this context, the effect of LY294002 on the proliferation of breast cancer cells containing normal or mutant PTEN was also examined. As the IC50 for LY294002 for purified PI3K activity is 1.44 μM (Vlahos *et al*., 1994), these studies were carried out using 1 μM LY294002. As indicated in Figure 8, cells which lack PTEN (BT-549 and MDA-MB-468) were markedly more sensitive to the growth inhibitory effects of LY294002 than were cells expressing functional PTEN (SKBr3 or MDA-MB-231). Together, these observations suggest that molecular therapeutics aimed at inhibiting the PI3K

pathway may be particularly efficacious in breast cancer cells lacking functional PTEN.

Discussion

In the current study, we have shown that breast cancer cells lacking PTEN protein demonstrate increased basal levels of PI3K activity and markedly increased duration of ligand-induced PI3K signaling. These findings suggest a role for PTEN in inhibiting both constitutive and ligand-induced PI3K signaling. This effect of PTEN is dependent on its phosphatase activity and more specifically on its lipid phosphatase activity as PI3K signaling is not influenced by the C124S and G129E mutant forms of PTEN, which ablate total phosphatase activity and only lipid phosphatase activity, respectively. While both PTEN and the PI3K inhibitor wortmannin decreased basal phosphorylation of AKT, wortmannin blocked ligand-induced AKT phosphorylation as efficiently as basal AKT phosphorylation. By contrast, PTEN had little effect on the magnitude of ligand-induced PI3K signaling. In breast cancer cells expressing functional PTEN protein, induced expression of additional PTEN did not alter basal or ligand-induced PI3K signaling (Figure 3). Taken together, these data suggest that PTEN is active constitutively and that its activity following cellular stimulation is constrained by an as of yet unknown mechanism so as to facilitate signaling through the PI3K pathway. Signal transduction may then be terminated, at least in part, by a subsequent return of PTEN activity to basal (high) levels. As PTEN dephosphorylates membrane phosphatidylinositols, this restoration of PTEN activity may reflect recruitment of PTEN to the membrane and/or changes in PTEN specific activity.

Considerable controversy exists as to the effects of PTEN on cell proliferation, viability and anoikis (Cheney *et al*., 1998, 1999; Davies *et al*., 1998, 1999; Furnari *et al*., 1997, 1998; Li and Sun, 1998; Li *et al*., 1998; Stambolic *et al*., 1998; Podsypanina *et al*., 1999; Ramaswamy *et al*., 1999; Sun *et al*., 1999; Tamura *et al*., 1999; Tian *et al*., 1999). In the current study, induced expression of PTEN in PTEN deficient breast cancer cells, was associated with a marked decrease in the basal phosphorylation of AKT, p70S6 kinase, BAD, and GSK3α, alterations indicative of decreased activity and signaling through the PI3K pathway. However, the effects of PTEN expression on ligand-induced activation of these molecules were limited. As AKT, BAD, p70S6 kinase and GSK3α have all been implicated in the regulation of apoptosis (Cardone *et al*., 1998; Datta *et al*., 1997; Delcommenne *et al*., 1998; Li and Sun, 1998; Pap and Cooper, 1998; Shi *et al*., 1995), these data suggest that expression of PTEN in the absence of exogenous ligand predisposes cells to undergo apoptosis. Further, expression of PTEN did not alter ligand-induced phosphorylation of *erk*2 indicative of intact signaling through the RAS-MAPK cascade which can prevent apoptosis (Xia *et al*., 1995; Werner and Le Roith, 1997; Downward, 1998). These observations may contribute to the finding that PTEN expression induces cellular apoptosis under low serum culture conditions, but not in the context of high serum concentrations (Figure 7). By contrast, the effects of PTEN on expression of the p27Kip1 CDK inhibitor was most clearly manifest in the presence of high serum concentrations (Figure 4) and, under these culture conditions, PTEN induced a partial G1 arrest (Figure 6 and Table 1). In the presence of serum, where cell survival is mediated by ligand dependent activation of the PI3K pathway or of the RAS/MAPK cascade (Figures 2 and 3), the increased expression of p27Kip1 is likely sufficient to mediate the partial growth arrest. Taken together, these data suggest that the growth inhibition engendered by PTEN reflects induction of both cell cycle arrest and apoptosis. Whether the differential effects of PTEN on basal versus ligand-induced signaling accounts for the disparities in the literature in relation to observed effects of PTEN on cell cycle progression and apoptosis remains to be determined. However, as previous studies of PTEN effects on cell cycle progression and apoptosis have utilized multiple different cell lineages and cell lines, the variability in study results may also reflect differences in the lineages and cell lines analysed.



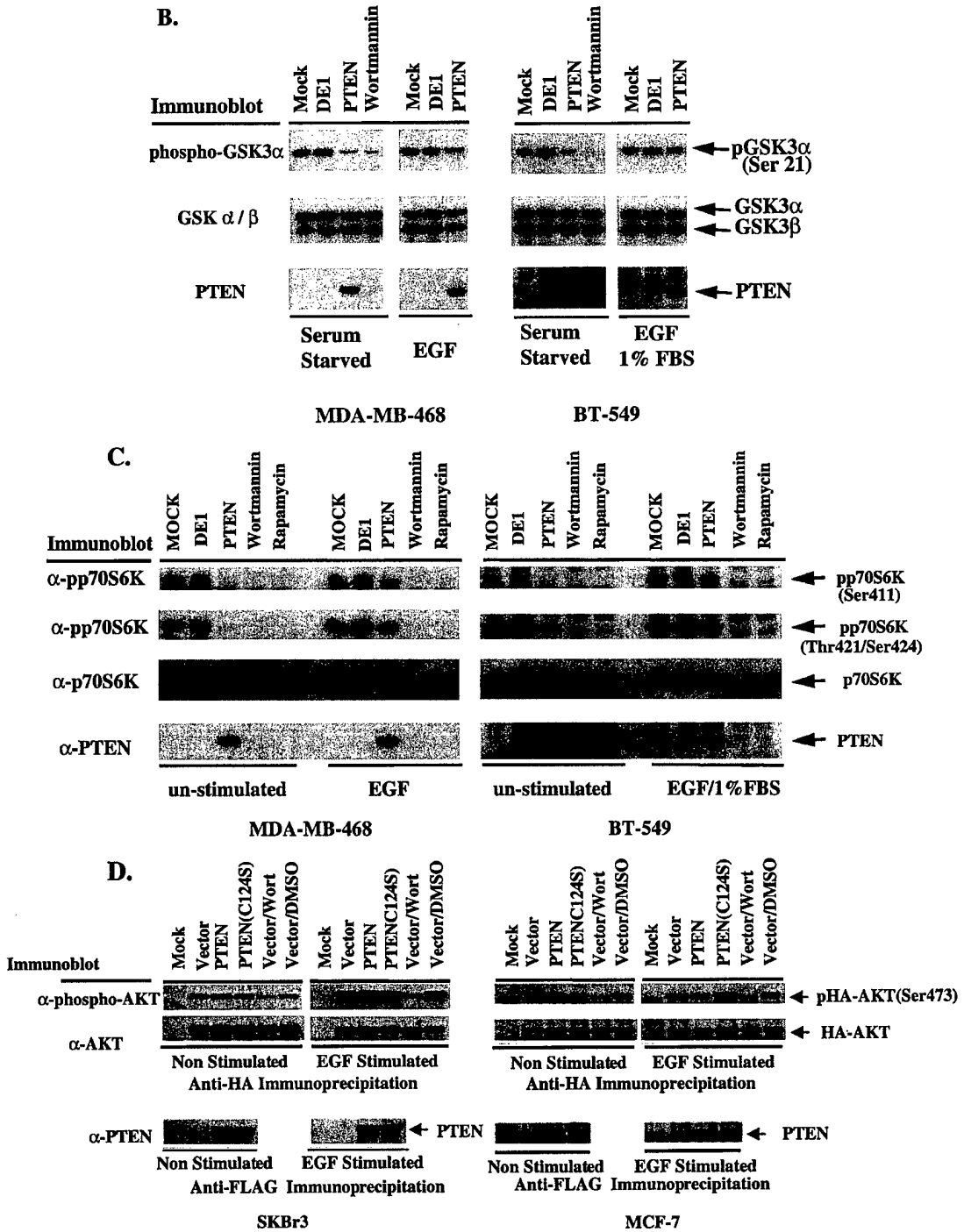


Figure 3 PTEN regulates phosphorylation of multiple components of the PI3K signaling cascade in PTEN mutant breast cancer cell lines. **(A)** BT-549 cells were cotransfected with AU-1 epitope-tagged BAD plus wild-type or C124S phosphatase inactive mutant of FLAG-PTEN. Twenty-four hours later cells were serum-starved overnight and stimulated with 1% FBS for 10 min. Total cellular proteins were separated by 11% SDS–PAGE and immunoblotted with anti-phospho-BAD Ser112 or Ser136. Membranes were re-probed with anti-AU-1 to confirm equal loading and transfection efficiency. **(B)** Total cellular proteins (30 μg) from adenoviral infected cells were separated by 11% SDS–PAGE and immunoblotted with anti-phospho-GSK3α. Membranes were re-probed with anti-GSK3α/β. To confirm the expression of PTEN, the membranes were re-probed with rabbit anti-PTEN antibody. **(C)** Total cellular proteins (30 μg) from adenoviral infected cells were separated by 8% SDS–PAGE and immunoblotted with anti-phospho-p70S6K Ser411 or Thr421/Ser424. Membranes were re-probed with anti-p70S6K or anti-PTEN to confirm equal loading and expression of PTEN. **(D)** Anti-HA immunoprecipitates of transfected cells were separated by 8% SDS–PAGE and immunoblotted with anti-phospho-AKT (Ser473). Membranes were re-probed with anti-AKT to confirm equal loading and transfection efficiency. Anti-FLAG immunoprecipitates were immunoblotted with rabbit anti-PTEN to confirm expression of PTEN

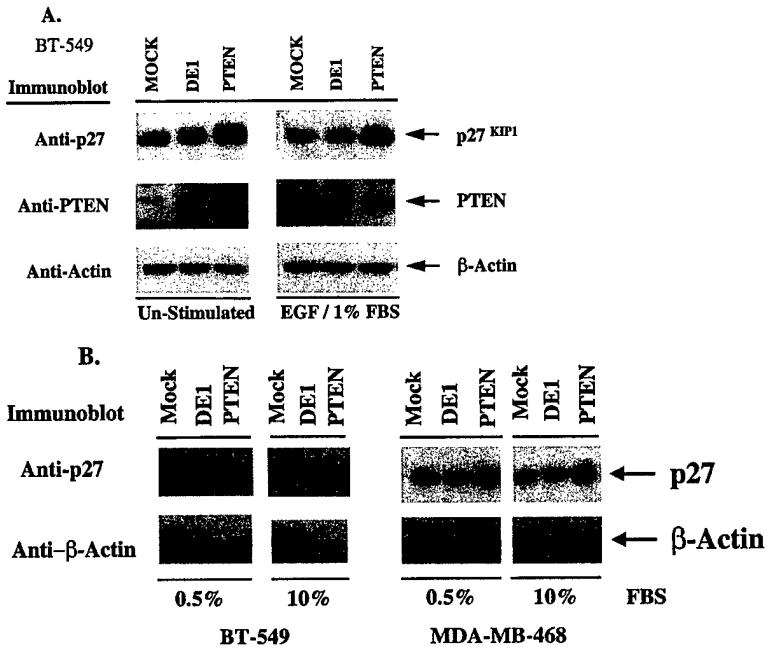


Figure 4 Effect of PTEN on protein levels of p27Kip1 in PTEN mutant breast cancer cells. Cells were mock infected or infected with adenovirus containing wild-type PTEN or empty virus (DE1) at 25 MOI. (**A**) Cells were cultured in the medium containing 10% FBS for 24 h following infection. Cells were then serum starved overnight and stimulated for 10 min with EGF (20 ng/ml) plus 1% FBS. Equal amount of cellular proteins (30 μg) from each sample were separated by 11% SDS–PAGE and immunoblotted with anti-p27Kip1. The membranes re-probed with rabbit-anti-PTEN to confirm the expression of PTEN. Cellular protein loading was determined by re-probing the membranes with anti-β-actin. (**B**) After virus infection, cells were cultured in the medium containing 0.5% FBS or 10% FBS for 48 h. Cells were lysed and the cellular proteins were immunoblotted with anti-p27Kip1 or anti-β-actin

In view of data revealing several of the small molecule inhibitors of the PI3K cascade to realize their inhibitory effects both *in vitro* and *in vivo* (Dumont and Su, 1996; Schultz *et al*., 1995; Shi *et al*., 1995; Vlahos *et al*., 1994), the current data showing differential sensitivity of PTEN-negative versus PTEN-positive cells to these inhibitors may be exploited therapeutically. This possibility is particularly relevant to treatment of breast cancers as the frequency of PTEN mutations in these cancers suggest that the development and progression of these tumors is significantly influenced by the PI3K pathway. Indeed, PTEN-deficient breast cancer cells appear to be more sensitive to the effects of LY294002-mediated PI3K inhibition than were breast cancer cells with normal PTEN expression (Figure 8). Importantly, the involvement of the PI3K pathway in tumorigenesis may be realized by mechanisms other than mutation of the PTEN tumor suppressor gene. Thus, for example, we have recently demonstrated that amplification of the p110α subunit of PI3K to be a frequent event in ovarian cancer and shown that the phenotypes of cells with mutant PTEN and amplified PI3K are remarkably similar. Our data indicate that cells with either one of these genetic defects exhibit increased signaling through the PI3K pathway, resistance to apoptosis and increased sensitivity to LY294002 (Shayesteh *et al*., 1999). Thus altered function of the PI3K pathway may be a frequent source of oncogenicity in multiple cell lineages and, as such, represents an attractive target for molecular therapeutics of cancer.

Materials and methods

Antibodies and reagents

Rabbit anti-PTEN has been described previously (Davies *et al*., 1998). Goat anti-PTEN (N-terminal) and monoclonal anti-p27Kip1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-AKT, anti-phospho-BAD, and anti-phospho-p70S6K were from New England Biolabs (Beverly, MA, USA). Sheep anti-phospho-GSK3α (Ser21), monoclonal anti-GSK3α/β and HRP-conjugated rabbit anti-sheep IgG were from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal anti-p21Cip1/Waf1 was from Transduction Laboratories (Lexington, KY, USA). Monoclonal anti-HA was from the mouse hybridoma 12CA5 (Dr Su, Houston, TX, USA). Monoclonal anti-FLAG (M2) and wortmannin were from Sigma (St. Louis, MO, USA). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were from BioRad (Hercules, CA, USA). HRP-conjugated protein A was from Amersham (Arlington Height, IL, USA). Protein A or protein G conjugated sepharose 4B were from Pharmacia Biotech (Piscataway, NJ, USA). LY294002 was from Calbiochem (La Jolla, CA, USA).

Expression vectors and adenoviruses

FLAG epitope-tagged full-length wild-type PTEN and catalytic inactive PTEN (C124S) were from Drs Dixon and Maehama (Ann Arbor, MI, USA). The G129E (Gly129-Glu) PTEN mutation was generated by site-directed mutagenesis.

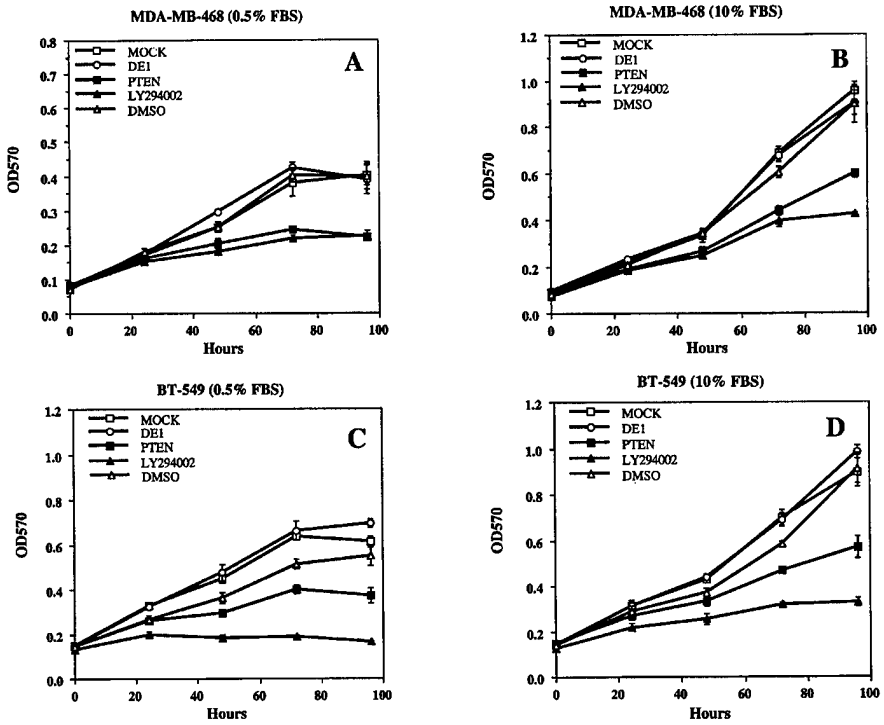


Figure 5 Expression of PTEN reduces cell growth in PTEN mutant breast cancer cells. Cells were cultured in 96-well plates (5000 cells/well) and mock infected or infected with adenovirus expressing (PTEN) or lacking (DE1) PTEN at 25 MOI. After infection, cells were cultured in medium containing 0.5% (**A** and **C**) or 10% FBS (**B** and **D**) for 96 h. Where indicated, mock infected cells were cultured in the presence of 10 μM LY294002 or vehicle (0.1% DMSO). Cell number and viability were assessed by MTT dye conversion and presented as mean of OD570. The data were from five replicates of a representative example of four similar experiments

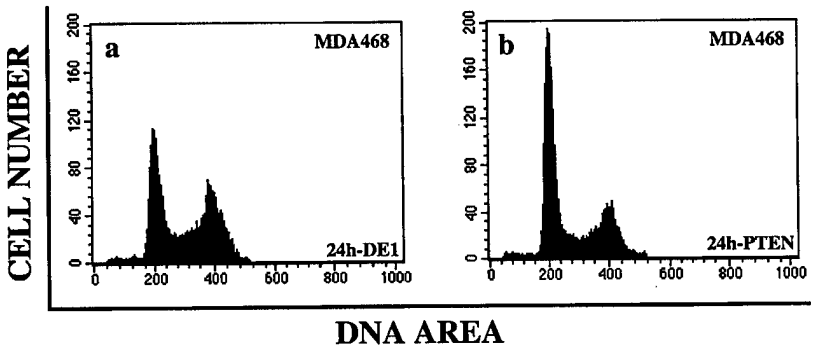


Figure 6 Expression of PTEN induces cell cycle arrest at G1. MDA-MB-468 cells were infected with adenovirus expressing PTEN and cultured in medium containing 10% FBS. Cell cycle distribution was determined by propidium iodine (PI) staining

Table 1 Per cent of cells distributed in cell cycle

Cell cycle	*<G0/G1*	*G0/G1*	*S*	*G2/M*
24 h DE1	1.83±0.09	39.80±2.05	34.47±2.02	24.73±4.06
PTEN	1.76±0.17	56.50±0.57	24.70±2.33	18.80±0.57
48 h DE1	2.47±0.17	37.33±2.00	35.73±3.40	25.20±2.14
PTEN	2.46±0.48	51.83±0.77	24.90±0.80	22.70±1.20

MDA-MB-468 cells were infected with adenovirus and treated as described in Figure 6. Per cent of cells distributed at various stages of cell cycle was measured and calculated by flow cytometry. Each treatment was performed in triplicate and presented as mean±s.e.m.

HA-epitope tagged wild-type AKT was from Dr Downward (UK). AU-1 tagged wild-type BAD was from Dr Gabriel Nunez (Ann Arbor, MI, USA). The recombinant adenovirus containing wild-type PTEN (Ad-MMAC) and empty adenovirus (Ad-DE1) were described previously (Davies *et al*., 1998; Liu *et al*., 1999).

Cell lines

Tumor cell lines were cultured in complete medium (RPMI 1640 supplemented with 10% FBS, and 2 mM L-

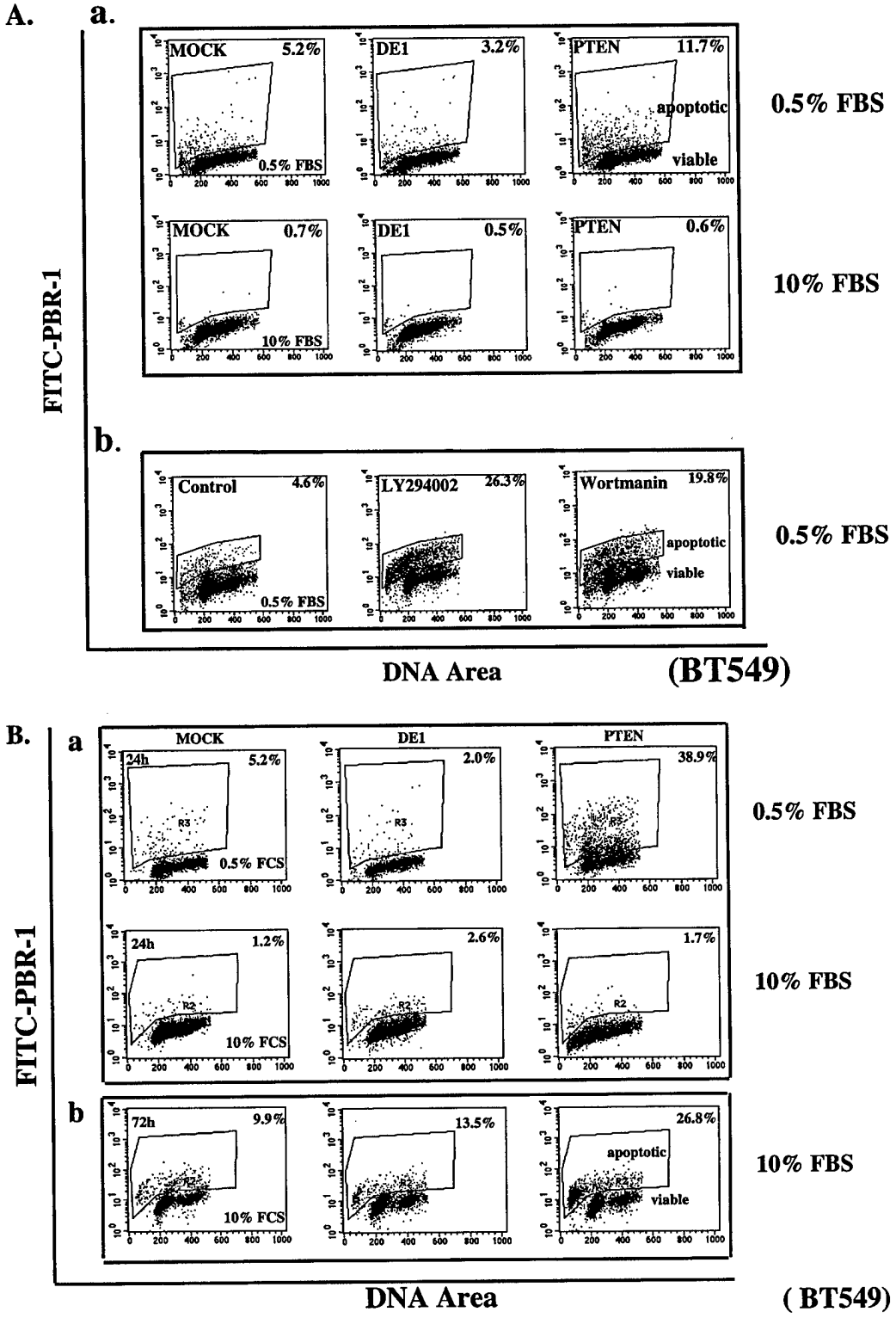


Figure 7 Expression of PTEN induces apoptosis and anoikis in PTEN mutant breast cancer cells. BT-549 cells were infected with adenovirus expressing PTEN. (**A**) Cells were cultured in serum restricted (0.5% FBS) or serum supplemented (10% FBS) conditions (a) or cultured in the presence or absence of 10 μM LY294002 or 1 μM wortmannin in serum restricted (0.5% FBS) conditions (b). Apoptosis was assessed at 72 h by APO-BRDU staining. (**B**) Forty-eight hours after viral infection, cells were collected and viable cells were cultured in serum restricted (0.5% FBS) or supplemented (10% FBS) conditions on a rocker. Apoptosis was assessed 24 h or 72 h later as indicated

glutamine). Immortalized normal breast epithelial cells MCF 10A and MCF 10F were cultured in DMEM/F12 (1:1) medium supplemented with 5% Equine Serum, Fungizone (0.5 μg/ml), Insulin (10 μg/ml), EGF (10 ng/ml), Cholera Endotoxin (100 ng/ml), Hydrocortisone (0.5 μg/ml) and $CaCl_2$ (1 mM).

Transient transfection and viral infection

Cells were transiently transfected with Fugene™ 6 Transfection Reagent (Boehringer Mannheim Inc., Indianapolis, IN, USA). For viral infection, cells were incubated with adenovirus at 25 MOI in a small volume of medium at 37°C for 1 h and then changed to complete medium.

Cell lysis, immunoprecipitation and immunoblotting

Cells were washed twice with PBS and lysed in ice-cold lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM Na Pyrophosphate, 1 mM Na_3VO_4, 10% glycerol, 1 mM PMSF and 10 μg/ml aprotonin). Cellular protein concentration was determined by BCA reaction (Pierce, Rockford, IL, USA). For immunoprecipitation, detergent lysates were incubated with 1 μg of anti-HA monoclonal antibody or 8 μg of anti-FLAG (M2) for 90 min. Immune complexes were captured by protein A sepharose beads (anti-HA) or protein G sepharose beads (anti-Flag). Immunoprecipitates were washed with 0.5% Triton X-100, 0.5% NP-40, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF. Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% BSA and then incubated for 2 h at room temperature or overnight at 4°C with anti-phospho-AKT (1:1000 dilution), AKT antibody (1:1000 dilution), anti-phospho-BAD Ser112 (1:1000 dilution), anti-BAD antibody (1:1000 dilution) or anti-phospho-BAD Ser136 antibody (1:500 dilution), anti-phospho-GSK3α (2 μg/ml), anti-GSK3α/β (0.5 μg/ml) antibody, anti-phospho-p70S6K (1:1000 dilution), anti-p70S6K (1:1000 dilution) antibody, anti-p27Kip1 mAb (1 μg/ml) or anti-p21Cip1/Waf1 mAb (1:500 dilution). Membranes were washed in TBS-T (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:2500 dilution) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence detection (ECL, Amersham).

Table 2 Expression of PTEN induces apoptosis in breast cancer cells

Infection	*APO2.7*	*Tunel*
0.5% FBS		
Mock	7.3	12.4
DE1	7.4	9.7
PTEN	21.9	41.0
10% FBS		
Mock	12.5	9.1
DE1	10.8	6.3
PTEN	14.2	6.7

BT-549 cells were infected with adenovirus expressing wild-type PTEN gene or empty virus (DE1) at 25 MOL. After infection, cells were cultured in the medium containing 0.5% or 10% of FBS for 48 h. Cells were harvested and fixed by 1% paraformaldehyde. Cells were then processed and analysed for apoptosis using the PE conjugated Apo2.7 (Clone 2.7A6A3) monoclonal antibody (Coulter Immunotech) or APO-BrdU Kit (Phoenix Flow System, Inc, San Diego, CA, USA) as recomended by the manufacturer

Cell growth assay

Cell growth was assessed by MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) dye conversion at 570 nm as described (Hansen *et al.*, 1989). Briefly, cells were seeded in 96-well plates (flat bottom, 5000 cells/well) and infected with adenovirus containing wild-type PTEN or empty virus (DE1). At each time point, 25 μl of MTT (5 mg/ml in PBS) was added to each well. After 2 h incubation at 37°C, cells were lysed by addition of 100 μl of 20% SDS in 50% DMF, pH 4.7.

Apoptosis and anoikis

Apoptosis and anoikis were measured using paraformaldehyde fixed cells with an APO-BrdU kit (Phoenix Flow Systems, Inc., San Diego, CA, USA) or binding of PE conjugated Apo 2.7 (Coulter Immunotech, Hialeah, Florida, USA) with flow cytometry. At each time point, both

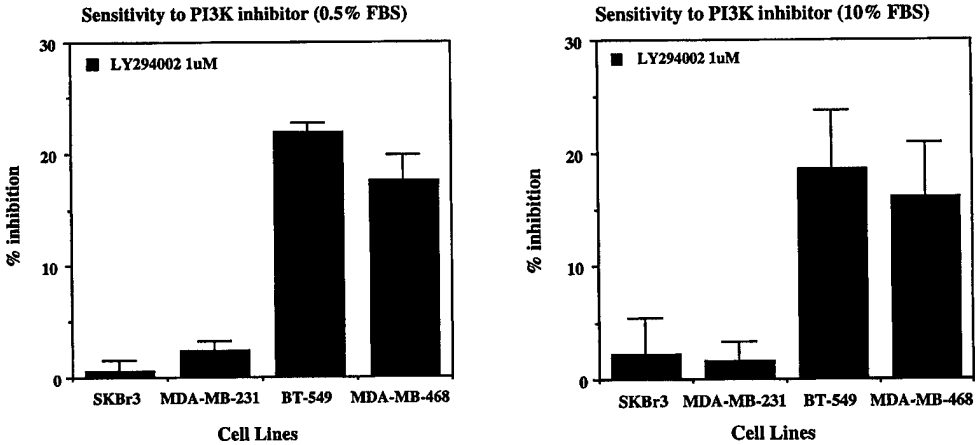


Figure 8 Breast cancer cells with mutant PTEN are more sensitive to growth inhibition by the PI3K inhibitor LY294002. Cells were cultured in 96-well plates (5000 cells/well) for 96 h in the presence or absence of LY294002 in medium containing 0.5% or 10% FBS. Cell number and viability were assayed by MTT dye conversion and presented as per cent inhibition [(OD570 without LY294002 – OD570 with LY294002)/OD570 without LY294002 × 100] (Mean ± s.e.m. for five replicates)

floating and attached cells were collected and washed with PBS. For anoikis assays, cells were incubated on a rocker platform.

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SHP-1 Regulates Lck-induced Phosphatidylinositol 3-Kinase Phosphorylation and Activity*

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Ligation of the T cell antigen receptor (TCR) activates the Src family tyrosine kinase p56 Lck, which, in turn, phosphorylates a variety of intracellular substrates. The phosphatidylinositol 3-kinase (PI3K) and the tyrosine phosphatase SHP-1 are two Lck substrates that have been implicated in TCR signaling. In this study, we demonstrate that SHP-1 co-immunoprecipitates with the p85 regulatory subunit of PI3K in Jurkat T cells, and that this association is increased by ligation of the TCR complex. Co-expression of SHP-1 and PI3K with a constitutively activated form of Lck in COS7 cells demonstrated the carboxyl-terminal SH2 domain of PI3K to inducibly associate with the full-length SHP-1 protein. By contrast, a truncated SHP-1 mutant lacking the Lck phosphorylation site (Tyr^{564}) failed to bind p85. Wild-type but not catalytically inactive SHP-1 induced dephosphorylation of p85. Furthermore, expression of SHP-1 decreased PI3K enzyme activity in anti-phosphotyrosine immunoprecipitates and phosphorylation of serine 473 in Akt, a process dependent on PI3K activity. These results indicate the presence of a functional interaction between PI3K and SHP-1 and suggest that PI3K signaling, which has been implicated in cell proliferation, apoptosis, cytoskeletal reorganization, and many other biological activities, can be regulated by SHP-1 in T lymphocytes.

In the context of appropriate co-stimulatory signals, ligation of the T cell antigen receptor (TCR)[1] by antigenic peptide bound to a major histocompatibility complex molecule leads to T cell activation and ultimately, a functional immune response. Activation of protein tyrosine kinases and consequent intracellular protein phosphorylation are among the first events elicited by TCR ligation and are crucial to the induction of biochemical pathways that regulate cell growth (1). This protein-tyrosine kinase activity, together with opposing protein-tyrosine phosphatase activity, plays a major role in regulating the magnitude of TCR-induced tyrosine phosphorylation, as well as the duration and termination of cell activation (1, 2). The counterbalance of tyrosine kinases by tyrosine phosphatases is integral to the maintenance of cellular homeostasis (3, 4), and disruption of this balance has been shown to be a hallmark of cellular transformation (5).

P56 Lck is a member of the Src family of non-receptor tyrosine kinases which is highly expressed in T lymphocytes (6). Along with the Fyn Src family kinase and the ζ-associated protein 70 (ZAP-70), Lck has been implicated in the initial activation events resulting from TCR ligation (1, 2, 6). Lck has been shown to associate with the CD4 and CD8 T cell surface antigens (6), and to play an integral role in the ligand-induced phosphorylation of the TCR intracellular components (1, 2, 6). Indeed, Lck-mediated phosphorylation of the ζ subunit of the TCR and ZAP-70 couples TCR ligation to a variety of downstream signaling molecules (2), and the loss of Lck activity significantly reduces the capacity of the TCR to transduce activation signals (7).

SHP-1 is an SH2 domain-containing non-receptor tyrosine phosphatase implicated in the negative regulation of a number of growth factor receptors, including the B and T cell antigen, erythropoeitin, the platelet-derived growth factor (PDGFR), c-kit, and the granulocyte macrophage colony-stimulating factor receptors (8–13). SHP-1 is highly expressed in T cells (4), and has also been linked to the negative regulation of TCR signaling (14–16). This effect of SHP-1 appears to reflect its capacity to down-regulate ZAP-70 (14) and Lck (17) activities and to also dephosphorylate TCR components and downstream signaling molecules (15, 16). SHP-1 has been shown to undergo tyrosine phosphorylation in response to CD4 or CD8 stimulation as well as Lck activation (18). As is consistent with an inhibitory effect of SHP-1 on TCR signaling, thymocytes from SHP-1-deficient viable motheaten exhibit a significantly increased proliferative response to stimulation by anti-CD3 antibodies as compared with normal mouse thymocytes (16, 17).

Ligation of the TCR alters inositol lipid metabolism through induction of phosphatidylinositol 3′-kinase (PI3K) activity (1). PI3K consists of a p85 regulatory subunit with two SH2 domains and a SH3 domain, and a p110 catalytic subunit which phosphorylates the 3′-hydroxyl of the inositol ring of phosphatidylinositol (19, 20). The resulting PI3K products bind to pleckstrin homology (PH) domains of intracellular signaling molecules recruiting them to the cell membrane. Activation of the PH domain containing c-Akt (21, 22) has been associated with cell cycle progression (23, 24) and the propagation of an anti-apoptotic signal (22, 25–27). Jurkat T cell activation via anti-CD3 antibody binding to the TCR complex has been shown to result in the rapid phosphorylation of both PI3K subunits

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[1] The abbreviations used are: TCR, T cell antigen receptor; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; HAp85, hemagglutinin epitope tag-labeled p85 construct; SH2, Src homology domain 2.

(28), as well as an accumulation of PI3K products (29). TCR-induced tyrosine phosphorylation of Tyr^{688} in the p85 subunit of PI3K and the consequent activation of PI3K have been linked to the presence of Lck (28, 30), and other recent data provide additional evidence of a role for Lck in PI3K signaling (31). However, the phosphatase(s) that dephosphorylates PI3K has not been identified as of yet.

In this study, we demonstrate that Lck activity is associated with an interaction of SHP-1 with the p85 subunit of PI3K, and also identify p85 as a target for SHP-1-mediated dephosphorylation. The association between p85 and SHP-1 requires tyrosine phosphorylation of SHP-1 and likely involves binding of SHP-1 phosphotyrosine 564 to the p85 carboxyl-terminal SH2 domain via a novel tyrosine recognition motif. This interaction is also associated with a reduction in the lipid kinase activity in total anti-phosphotyrosine immunoprecipitates and a reduction in PI3K-mediated phosphorylation of Akt. Together, these findings implicate the interaction of SHP-1 with PI3K in the modulation of the PI3K signaling cascade downstream of TCR engagement.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—A monoclonal antibody against the ϵ chain of human CD3 complex (UCHT1, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Peter Beverly (University College, London, United Kingdom). The rabbit polyclonal antibody against Lck was described previously (32). The anti-phosphotyrosine monoclonal antibody (4G10, IgG1) and the rabbit polyclonal antibody against the p85 subunit of PI3K, and the rabbit polyclonal antibody against SHP-1 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against Akt and phospho-Akt were purchased from New England Biolabs (Beverly, MA). A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas at Houston). Rabbit anti-mouse IgG was purchased from Western Blotting Inc. (Toronto, ON). Horseradish peroxidase goat anti-mouse IgG was purchased from Bio-Rad. Glutathione-Sepharose and protein A-Sepharose beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). GST fusion proteins of the p85 SH2 domains were generous gifts of Dr. T. Pawson (Toronto, ON). The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Veillette (Montreal, QE). The cDNA plasmid for HAAkt was a generous gift of Dr. Julian Downward (London, United Kingdom). The cDNA plasmid for HAp85 and ΔHAp85 were described previously (33).

Cell Lines—Human Leukemic Jurkat T cell line E6.1, and COS7 cells were purchased from American Type Culture Collection (Rockville, MD).

Cell Culture, Stimulation, and Lysis—Jurkat T and COS7 cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) containing penicillin/streptomycin (1%, Life Technologies, Inc.), L-glutamine (2 mM, Life Technologies, Inc.), and 10% (v/v) fetal calf serum (Sigma) at 37 °C in a humidified atmosphere. For CD3 cross-linking, cells were incubated with anti-CD3 (0.6 μg/ml) antibodies plus rabbit anti-mouse IgG (10 μg/ml) at room temperature for the indicated time periods. After stimulation, the cells were pelleted, resuspended in 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.4, 1 mM sodium orthovanadate, 50 mM $ZnCl_2$, 50 mM sodium fluoride, 50 mM sodium orthophosphate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and incubated at 4 °C for 20 min. After centrifugation at 14,000 × *g* for 5 min at 4 °C, post-nuclear detergent cell lysates were collected.

Transient Transfection—COS7 cells were transfected by Lipofection. Briefly, 4×10^5 cells were seeded on 100-mm cell culture plates and incubated in complete media overnight. cDNA expression constructs were incubated in serum-free medium with LipofectAMINE (Life Technologies, Inc.) at room temperature for 30 min, then diluted with serum-free medium and incubated with cells at 37 °C for 2 h, after which time the LipofectAMINE mixture was replaced with complete media and the cells were returned to 37 °C for 24 h. Complete media was then removed, the cells rinsed, and incubation continued with serum-free medium for an additional 24 h.

Immunoprecipitation and Immunoblotting—Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4 °C for 2 h followed by another 2-h incubation with protein A-Sepharose beads. The immunoprecipitates were washed with IP wash buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), anti-phosphotyrosine (1:3000), or anti-SHP-1 (1:400) at room temperature for 2 h. Horseradish peroxidase-protein A or horseradish peroxidase-goat anti-mouse IgG was used as the secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL, Amersham). Where indicated, blots were stripped by treatment with 2% SDS and 100 mM β-mercaptoethanol in Tris-buffered saline and then reprobed with anti-p85 PI3K antibodies and detected by ECL.

Fusion Protein Binding Assays—Transfected COS7 cells were starved for 24 h in serum-free medium. The cells were lysed in Nonidet P-40 lysis buffer. Bacterial lysates containing the fusion protein GST alone, the p85 amino-terminal SH2 domain, or the p85 carboxyl-terminal SH2 domain were diluted in phosphate-buffered saline and incubated with glutathione-Sepharose beads. GST fusion protein beads were washed, then incubated with the transfected cell lysate at 4 °C for 2 h. After extensive washing, the proteins were eluted and immunoblotted as described above.

Kinase Activity—Cells were lysed in 1% Nonidet P-40 lysis buffer. Cell lysates normalized for protein levels (BCA assay; Pierce Chemical Co., Rockford, IL) were immunoprecipitated using anti-HA and protein A-Sepharose. Non-transfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described (34). Briefly, the immunoprecipitates were washed sequentially in: (*a*) phosphate-buffered saline, 100 μM Na_3VO_4, 1% Triton X-100; (*b*) 100 mM Tris, pH 7.6, 0.5 M LiCl, 100 μM Na_3VO_4; (*c*) 100 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 100 μM Na_3VO_4; (*d*) 20 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 30 mM $NaPP_i$, 200 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.03% Triton X-100, and resuspended in 30 μl of kinase reaction buffer (33 μM Tris, pH 7.6, 125 mM NaCl, 15 μM $MgCl_2$, 200 μM adenosine, 15 μM ATP, 30 μCi of [γ-^{32}P]ATP). Phosphatidylinositol (PI) was resuspended in 20 mM Hepes, pH 7.5, at 2 mg/ml and sonicated on ice for 10 min. The PI 3-kinase reaction was initiated by adding 10 μl of the PI suspension. The reaction proceeded for 30 min at room temperature and was terminated by adding 100 μl of 1 N HCl. Lipids were extracted by 600 μl of chloroform:methanol (1:1). The organic phase was washed with H_2O, collected and dried by vacuum centifugation. The lipids were resuspended in 20 μl of chloroform:methanol (1:1) and resolved on Silica Gel G-60 thin-layer chromatography (TLC) plates in cloroform:methanol:NH_4OH:H_2O (60:47:2:11.3). Radiolabeled phosphatidylinositol phosphate was visualized by autoradiography.

Lck Autophosphorylation Assay—Cells were lysed in kinase lysis buffer (35). Cell lysates normalized for protein levels were immunoprecipitated using a rabbit antibody against human Lck and protein A-Sepharose. Non-transfected COS7 and SHP-1 transfected cell lysates were used as negative controls. After immunoprecipitation, the beads were washed four times with wash buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Hepes, pH 7.5, 1 mM Na_3VO_4). The washed beads are then resuspended in 50 μl of kinase reaction mixture (20 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 μM ATP, 10 μCi of [γ-^{32}P]ATP) and incubated at room temperature for 30 min. The reaction was stopped by washing the beads twice with wash buffer including 1 mM EDTA. Proteins were eluted from the beads by boiling in 2 × Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA). Radiolabeled Lck was visualized by autoradiography.

Subcellular Fractionation—Jurkat cells were incubated in serum-free RPMI for 16 h prior to stimulation. Cells were divided into two aliquots (25×10^6 cell each), and one was stimulated by cross-linking TCR complex proteins with anti-CD3 (see above) for 7 min. Membrane and cytosolic fractions were separated based on the protocol of Resh and Erickson (36). Briefly, cells were washed twice with STE (150 mM NaCl, 50 mM Tris, 1 mM EDTA) and collected with low speed centrifugation (1,000 × *g*). The cells were resuspended in hypotonic lysis buffer (10 mM Tris, 0.2 mM $MgCl_2$, 5 mM KCl, 1 mM $NaVO_4$, pH 7.4) and incubated on ice for 15 min. The cells were lysed with 30 strokes in a Dounce homogenizer. Lysates were adjusted to 0.25 M sucrose, 1 mM EDTA, and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatant was removed, and the pellet resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and given five additional strokes in a Dounce homogenizer, and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatants were combined and centifuged at 100,000 × *g* for 1 h. The result-

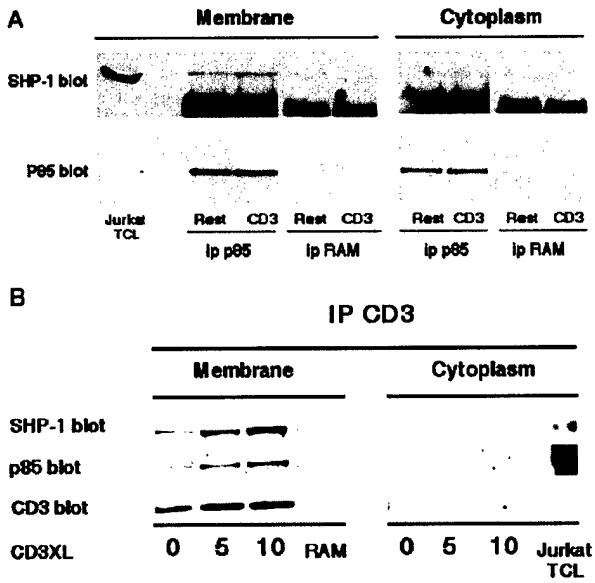

FIG. 1. **SHP-1 co-immunoprecipitates with p85 regulatory subunit in Jurkat cells.** Jurkat T cells were stimulated with anti-CD3 antibodies, lysed, and subjected to subcellular fractionation. *A*, equivalent amounts of protein from resting and stimulated fractions were immunoprecipitated with antibody to p85 and the precipitated proteins then subjected to immunoblotting with anti-SHP-1 antibody. The p85 immunoblot (*lower panel*) demonstrates equal loading of the test samples. An identical set of samples was immunoprecipitated (*ip*) with rabbit anti-mouse (*RAM*) antibody as a control. Data are representative of three independent experiments. *B*, membrane fraction was immunoprecipitated with anti-CD3 antibody and the precipitated proteins subjected to immunoblotting with anti-SHP-1 and anti-p85 antibodies, with an anti-CD3 immunoblot performed to demonstrate equal loading.

ing supernatant was labeled S100 (cytosolic), and the pellet labeled P100 (membrane). The P100 fraction was resuspended in phosphate-buffered saline. All samples were pre-cleared with protein A-Sepharose for 1 h at 4 °C. Both fractions were divided into two samples each, with one sample to be immunoprecipitated with anti-p85 antibody, and the other with rabbit anti-mouse antibody as a negative control.

RESULTS

SHP-1 Physically Associates with PI3K—Although PI3K has been shown to be phosphorylated and activated following TCR ligation (28), the phosphatase responsible for dephosphorylation of PI3K has yet to be identified. The tyrosine phosphatase SHP-1 has been shown to target a number of molecules required for TCR signal relay (4). To address the possibility that PI3K represents a SHP-1 target, the capacity for SHP-1 to associate with PI3K in TCR-stimulated Jurkat cells was investigated by cross-linking the TCR complex with antibodies to CD3. We utilized a subcellular fractionation approach (36) to maximize the yield of activated, membrane-associated PI3K and reduce dilution by non-activated PI3K. Results of immunoblotting analysis indicated SHP-1 to be present in p85 immunoprecipitates from the membrane fraction (Fig. 1*A*, representative of three experiments) but not the cytosolic fractions of Jurkat T cells. TCR ligation resulted in a doubling, as assessed by densitometric analysis, of the amount of SHP-1 associated with p85 (Fig. 1, *lanes 1* and *2*), a result which is suggestive of recruitment of SHP-1 to a complex containing PI3K upon activation. Compatible with the presence of SHP-1 in PI3K immunoprecipitates, CD3 ligation induced a time-dependent increase in the amount of SHP-1 and PI3K present in membrane fraction anti-CD3 immunoprecipitates (Fig. 1*B*). The similar kinetics of association of SHP-1 and p85 with the TCR place these two signaling proteins at the activated TCR at the same time, and provide further evidence of a complex containing both SHP-1 and PI3K. Thus SHP-1, both constitutively and inducibly, associates with membrane bound and presumably activated PI3K in Jurkat cells (19, 20, 37, 38), either directly or as part of a multimeric complex. Whether the baseline association of these proteins reflects constitutive activation of Jurkat cells, even in serum-free medium, remains to be determined.

The p85 Carboxyl-terminal SH2 Domain Binds Phosphorylated SHP-1—To determine the functional relationship between PI3K and SHP-1, we used a transient transfection system involving the expression of recombinant p85 and SHP-1 in COS7 cells. T cell receptor activation was simulated in this system by overexpression of a constitutively activated form of Lck (Lck Y505F) that was generated by mutating the regulatory carboxyl-terminal inhibitory tyrosine (6). In previous studies, the regulatory PI3K subunit p85 has been shown to be phosphorylated by Lck Y505F when these proteins are co-expressed in COS1 cells (30). The major site of Lck-induced p85 phosphorylation has been mapped to a tyrosine residue (Tyr^{688}) located within the carboxyl-terminal SH2 domain (30). As Tyr^{564} in the SHP-1 carboxyl-terminal tail is also phosphorylated by Lck, and both p85 and SHP-1 contain SH2 domains, Lck-induced physical association of p85 with SHP-1 might be mediated by binding of the p85 SH2 domain(s) to phosphotyrosine on SHP-1. Alternatively, the SH2 domain of SHP-1 might inducibly associate with phosphorylated p85. To distinguish between these possibilities, the capacity of GST fusion proteins containing the p85 amino- or carboxyl-terminal SH2 domains to precipitate SHP-1 from lysates of transfected COS7 cells was examined. For these studies, the cells were transfected with a catalytically inactive form of SHP-1 (SHP-1 C453S) so as to prevent autodephosphorylation (18) and thus maximize the level of SHP-1 phosphorylation. As illustrated by the anti-SHP-1 Western blot shown in Fig. 2*A*, the results of this analysis revealed only the carboxyl-terminal SH2 domain of p85 to bind SHP-1 C453S, and indicated this association to require the presence of Lck Y505F. By contrast, tyrosine-phosphorylated p85 was not precipitated by GST-SHP-1 SH2 domain fusion proteins (data not shown). To determine whether the major site on SHP-1 for Lck-mediated phosphorylation (18) was involved in the p85 SH2-mediated association between p85 and SHP-1, a truncation mutant construct (SHP-1 Δ35) encoding amino acids 1 through Lys^{560} of SHP-1 and thus lacking Tyr^{564}, was derived and its capacity to associate with the p85 carboxyl-terminal SH2 domain then examined in the transfected COS7 cells. As illustrated by Fig. 2*B*, immunoblot analysis revealed the failure of SHP-1 Δ35 to associate with the p85 carboxyl-terminal domain, and thus demonstrated this association to require one or more amino acids mapping within the Δ35 segment. As Tyr^{564}, located within the last 35 amino acids of SHP-1, is the primary site of Lck phosphorylation in SHP-1, and Lck is required for the association of SHP-1 with the carboxyl-terminal SH2 domain of PI3K (Fig. 2*A*), these data strongly suggest that it is the interaction of this phosphorylated residue with the p85 carboxyl-terminal SH2 domain which mediates physical association of p85 with SHP-1.

SHP-1 dephosphorylates Lck-phosphorylated p85—Association of the p85 SH2 domain with the carboxyl terminus of SHP-1 creates the opportunity for SHP-1 to dephosphorylate Tyr^{688} of p85 (Fig. 2*C*), the major site of Lck phosphorylation on p85 (30). Accordingly, the possibility that SHP-1 dephosphorylates Lck-phosphorylated p85 was investigated in COS7 cells co-transfected with a recombinant hemagglutinin epitope tag-labeled p85 construct (HAp85), Lck Y505F, and SHP-1. Immunoprecipitation of HAp85, followed by SDS-PAGE and Western blotting with anti-phosphotyrosine clearly demonstrate the co-

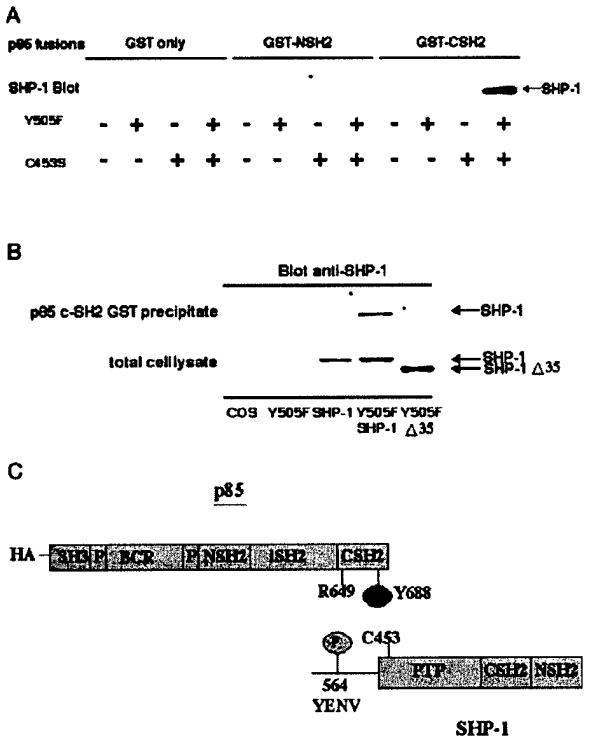

FIG. 2. **The p85 carboxyl-terminal SH2 domain binds phosphorylated SHP-1.** COS7 cells were transfected, then expanded in culture for 48 h prior to cell lysis. *A*, COS7 cells were transfected with SHP-1 C453S, Lck Y505F, or with both SHP-1 C453S and Lck Y505F. Lysates were mixed with either GST alone or GST-p85 SH2 fusion proteins immobilized on glutathione-agarose beads. Bound proteins were separated by SDS-PAGE and transferred to an Immobilon membrane. Bound SHP-1 C453S was detected by probing the membrane with anti-SHP-1. *B*, SHP-1 Δ35 transfected COS7 cell lysate is included as a test sample in a repeat of the GST-p85 carboxyl-terminal SH2 fusion protein binding assay. Data are representative of three independent experiments. *C*, a schematic depicting the proposed model of SHP-1 association with p85 is shown.

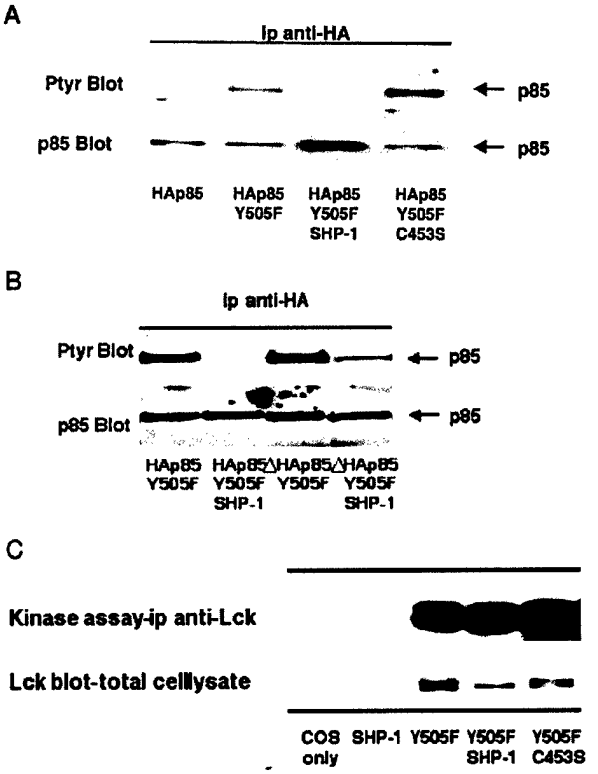

FIG. 3. **SHP-1 dephosphorylates p85.** *A* and *B*, COS7 cells were transfected as indicated. HA epitope-tagged p85 was immunoprecipitated with anti-HA antibodies, and the proteins separated by SDS-PAGE followed by transfer to Immobilon. Tyrosine-phosphorylated p85 was detected by probing with anti-phosphotyrosine antibodies. Data are representative of four independent experiments. *C*, COS7 cells were transfected with Lck Y505F and SHP-1 or SHP-1 C453S, and lysed after 48 h. The lysates were immunoprecipitated with anti-Lck antibody and analyzed by Lck autokinase assay. Data are representative of three independent experiments.

transfection of HAp85 with Y505F to induce a level of tyrosine phosphorylation of the recombinant p85 protein which is significantly increased relative to the vector control (Fig. 3*A*, *lanes 1* and *2*). Expression of SHP-1 with Y505F and HAp85 in this system was associated with a reduction of p85 phosphorylation to a level comparable to that detected in vector control cells (Fig. 3*A*, *lanes 1* and *3*). Thus p85 appears to represent a SHP-1 substrate. Interestingly, substitution of wild-type SHP-1 with SHP-1 C453S not only restored p85 phosphorylation to the level detected in the Y505F/HAp85 lysate, but also engendered the highest p85 phosphorylation detected in any transfectant (Fig. 3*A*).

As p85 heterodimerizes with the p110 subunit of PI3K, the possibility that association with p110 was required for SHP-1-mediated dephosphorylation of p85 was also studied. To this end, the Lck Y505F transfected COS7 cells were also co-transfected with a mutant form of p85 (ΔHAp85) (39) in which the inter-SH2 (iSH2) p110-binding region, that is absolutely required for p85 heterodimerization (Fig. 2*C*), was deleted. Analysis of these cells revealed ΔHAp85 to be both phosphorylated by activated Lck, and dephosphorylated by SHP-1 (Fig. 3*B*). Thus, while the physical association between p110 and SHP-1 cannot be excluded, these data suggest that such an association is not necessary for the SHP-1-mediated dephosphorylation of p85.

Although the Lck Y505F mutant used in these studies lacks the regulatory carboxyl tyrosine, it is possible that the effects of SHP-1 on p85 phosphorylation relate to SHP-1-mediated dephosphorylation of other phosphotyrosine sites in Lck and consequent down-regulation of Lck Y505F activity. To assess this possibility, Y505F autophosphorylation *in vitro* was examined in COS7 cells transfected with Lck Y505F alone or in combination with either SHP-1 or SHP-1 C453S. The results of this assay revealed the *in vitro* kinase activity of Lck Y505F to remain intact in the presence of SHP-1 expression (Fig. 3*C*). Taken together, these data indicate that p85 not only physically associates with SHP-1, but also is dephosphorylated by SHP-1.

Effect of SHP-1 Expression on PI3K Activity—To determine whether SHP-1-mediated dephosphorylation of p85 is associated with a change in PI3K activity, epitope-tagged p85 was immunoprecipitated from COS7 co-transfectants and the kinase activity of the associated p110 catalytic subunit was evaluated using an *in vitro* lipid phosphorylation assay. The results of this analysis revealed PI3K lipid kinase activity to be unaffected by SHP-1 expression (data not shown). However, as SHP-1 interaction with PI3K involves PI3K tyrosine phosphorylation, the possibility that SHP-1 binding diminishes activity of phosphorylated, but not total cellular PI3K, was also addressed. To this end, anti-phosphotyrosine antibodies were used to immunoprecipitate phosphorylated proteins from the COS7 lysates, and the precipitated phosphoproteins were then

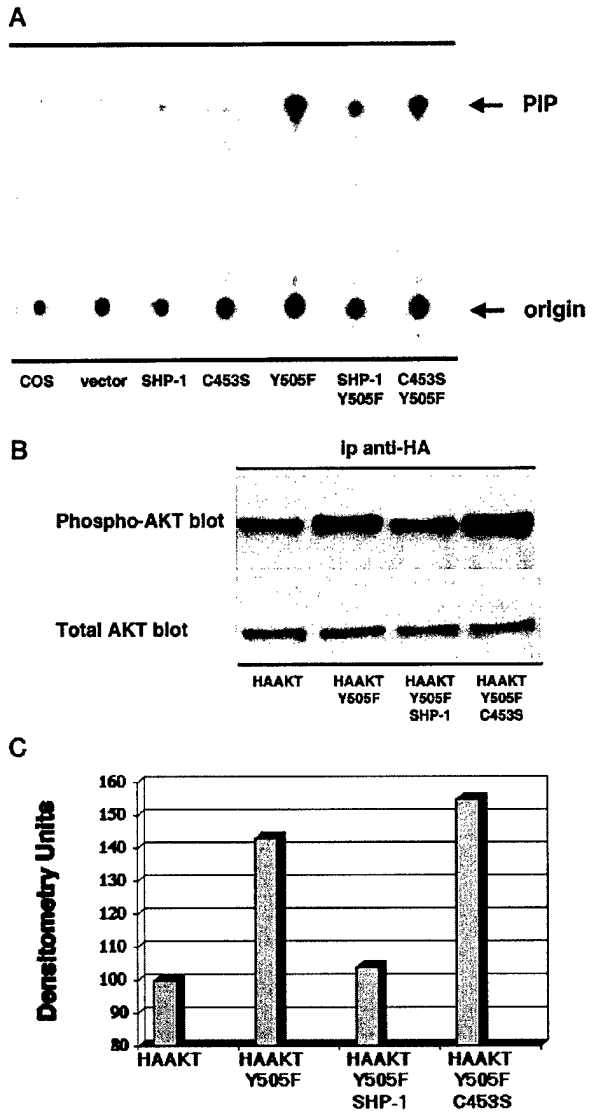

FIG. 4. **SHP-1 expression results in a decrease of PI3K activity.** *A*, COS7 cells were transfected as indicated previously. Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies, and then subjected to an *in vitro* lipid kinase assay. The assay mixture was separated on thin-layer chromatography plates, and 3′-phosphorylated lipids detected by autoradiography. *PIP*, phosphatidylinositol. *B*, anti-HA immunoprecipitates (*ip*) were separated and transferred to Immobilon and the filters probed with antibody to Ser^{473}-phosphorylated AKT. A subsequent immunoblot with antibody to total AKT was performed to demonstrate equal loading. *C* represents the densitometric variation of the co-transfected samples in *B* as compared with the sample transfected with HAAkt. HAAkt is arbitrarily set as 100. Data are representative of four independent experiments.

evaluated for lipid kinase activity. Results of this analysis revealed the lipid kinase activity present in the tyrosine-phosphorylated fraction to be markedly reduced in the Lck Y505F/SHP-1 co-transfectants as compared with the transfectants in which Lck Y505F was expressed in the absence of SHP-1 (Fig. 4*A*). By contrast, expression of SHP-1 C453S did not affect anti-phosphotyrosine immunoprecipitable lipid kinase activity, a result which indicates the decreased PI3K activity observed in the Lck Y505F/SHP-1 cells to be dependent on the phosphatase activity of SHP-1.

The regulatory effects of SHP-1 on PI3K signaling were also investigated by analyzing the relevance of SHP-1 to the activities of signaling molecules downstream of PI3K. Most notable among the latter proteins is Akt, a PH domain-containing kinase linked to cell cycle progression, proliferation, and cell death (40). Phosphorylation of Akt at serine residue 473 (S473) is absolutely dependent on PI3K activity (22), being abrogated by PI3K inhibitors LY294002 and wortmannin (data not shown). Evaluation of PI3K-dependent Akt Ser^{473} phosphorylation thus provides a surrogate assay for PI3K activity in intact cells. To explore the effects of SHP-1 on PI3K-induced Akt phosphorylation, hemagglutinin-tagged Akt (HAAkt) and Lck Y505F were co-transfected in COS7 cells and the phosphorylation of Akt examined by immunoblotting analysis using an anti-Akt antibody specifically recognizing phosphoserine 473. Results of this analysis (Fig. 4*B*) revealed Lck Y505F co-transfection to be associated with a modest increase in Akt Ser^{473} phosphorylation. By contrast, co-expression of wild-type SHP-1 with Lck Y505F and HAAkt reduced phospho-Akt to a level similar to that detected in cells transfected with HAAkt alone. Interestingly, expression of SHP-1 C453S in conjunction with Lck Y505F and HAAkt was associated with increases in levels of phospho-Akt exceeding those detected in cells expressing Lck Y505F and HAAkt (Fig. 4, *B* and *C*). These latter findings parallel the observations revealing Lck Y505F effects on p85 phosphorylation (Fig. 3*A*) to be somewhat enhanced in the context of SHP-1 C453S expression, a finding which suggests that substrate trapping by the latter protein may impact on PI3K signaling.

DISCUSSION

In the current study, the possibility that interaction between PI3K and SHP-1 contributes to the effects of these respective proteins on TCR signaling was investigated. The data reveal that SHP-1 interacts with the p85 subunit of PI3K in Jurkat T cells, and indicate this association to be enhanced by TCR stimulation. Furthermore, SHP-1 and PI3K are present in a complex including the TCR. Association of SHP-1 with p85 was also found to be inducible in COS7 cells by addition of activated Lck and to represent a phosphotyrosine-dependent interaction involving association of the p85 carboxyl-terminal SH2 domain likely with phosphorylated tyrosine 564 in the SHP-1 carboxyl-terminal tail. By further analysis of this interaction in COS7 cells, p85 was identified as a substrate for SHP-1, and the activity of tyrosine-phosphorylated PI3K shown to be markedly reduced in the presence of wild-type, but not catalytically inert SHP-1 (41). SHP-1 expression did not, however, alter lipid kinase activity of total cellular PI3K. A role for SHP-1 in regulating PI3K signaling was also evidenced by the finding that SHP-1 expression in COS7 cells engenders a decrease in phosphorylation of Akt Ser^{473}. Phosphorylation of Akt at this site involves association of the Akt PH domain with phosphorylated PI3K lipid substrates in the cell membrane and is known to be completely dependent on PI3K activation (22). Taken together, these observations provide evidence that SHP-1 not only interacts with PI3K, but also impacts upon PI3K activation and downstream signaling.

The current data indicate the SHP-1/PI3K interaction to be mediated by binding of the PI3K p85 subunit carboxyl-terminal SH2 domain to phosphorylated SHP-1 and to require that the most carboxyl-terminal located 35-amino acid segment of SHP-1 be intact. As Tyr^{564}, which has been identified as the primary target for Lck effects on SHP-1, maps within this region (18), it appears likely that Tyr^{564} represents the site on SHP-1 which interacts with the p85 SH2 domain. Interestingly, the results of these studies also revealed the truncated SHP-1 Δ35 protein to exhibit decreased phosphatase activity (data not shown), a result which contrasts with previous data

suggesting catalytic activity of this mutant form of SHP-1 to be enhanced (42). This discrepancy may reflect the differences in the conditions used for the respective phosphatase assays, the previous study involving analysis of PTP activity at pH 5.5. In the current study, the assay was performed at pH 7.3, which would presumably more closely approximate physiologic conditions. In any case, in view of the potential for this truncation mutation to alter SHP-1 activity, the SHP-1 Δ35 protein was used here only in binding studies, and its effects on p85 phosphorylation and PI3K activity were not examined.

Although p85 SH2 domains have been previously shown to specifically target YMXM phosphotyrosine motifs, the current data suggest that the carboxyl-terminal SH2 domain of p85 binds a SHP-1 phosphotyrosine residue (Tyr^{564}) embedded within a YENV motif. This divergence in the SH2 domain specificity is, however, not without precedent (30, 43). The SHP-1 SH2 domains, for example, have been demonstrated to interact with several distinct phosphotyrosine motifs (44). Furthermore, *in vitro* phosphorylation of the p85 carboxyl-terminal SH2 domain has been shown to alter its capacity to bind certain targets in activated Jurkat cells (30), a finding which again raises the possibility that the SH2 domain may interact with phosphotyrosines in more than one structural context.

Interestingly, p85 association with SHP-1 in PDGFR-stimulated MCF-7 cells has been shown to be mediated by binding of the SHP-1 amino-terminal SH2 domain to phosphorylated p85 (10). By contrast, interaction of the SHP-1 SH2 domains with phosphorylated p85 was not detected in the current study, a discrepancy which may reflect differences in the PI3K sites targeted by Lck and PDGFR, respectively (18, 45). It is also not clear whether p85 is a direct PDGFR target *in vivo*. However, taken together, these findings raise the possibility that association of SHP-1 with PI3K and the consequent modulation of PI3K signaling occurs in a variety of cell stimulatory contexts.

The data reported here concur with other data in the literature revealing the phosphorylation of p85 and the *in vitro* lipid kinase activity of immunoprecipitated PI3K to be poorly correlated (30). However, wild-type SHP-1 decreases PI3K activity in anti-phosphotyrosine immunoprecipitates and PI3K-dependent phosphorylation of Akt in intact cells. Interestingly, both p85 phosphorylation and PI3K activity, as revealed by Akt S473 phosphorylation, were found to be up-regulated in the presence of catalytically inactive SHP-1 C453S protein. As SHP-1 C453S does not enhance activity of Lck Y505F (Fig. 3*C*), these data suggest that SHP-1 C453S acts in this context as a "substrate trap," binding phosphorylated targets, but failing to dephosphorylate or release these phosphoproteins, thus protecting them from dephosphorylation by other cellular phosphatases. The increased level of phospho-Akt in the SHP-1 C453S-transfected cells may also reflect the capacity of mutant SHP-1 C453S protein bound to PI3K to impede PI3K interaction with a negative regulator of PI3K, or, alternatively, the capacity of PI3K bound SHP-1 C453S to induce conformational changes in PI3K which favor its activation, possibly by mimicking the effects of a positive modulator of PI3K. Both of these latter hypotheses suggest the involvement of a third molecule in the PI3K/SHP-1 interaction, a possibility also suggested by our finding that SHP-1 and PI3K can be co-immunoprecipitated from the membrane fraction of resting, serum-starved Jurkat cells in which protein phosphorylation would be expected to be minimal. Therefore, SHP-1 may also associate with PI3K by a phosphotyrosine-independent mechanism, such as interactions with an SH3 domain containing protein (46). This possibility however, remains purely speculative at present.

In summary, the data shown here reveal a functional relationship between Lck, SHP-1, and PI3K signaling proteins, which have each been identified as key elements in the induction of T cell activation. While Lck acts primarily to promote TCR signaling (6), SHP-1 effects on TCR signal relay are largely inhibitory (16, 17). The current data suggest that this inhibitory effect of SHP-1 is realized at least in part through the down-regulation of PI3K activity. However, in view of the limited understanding of the role for PI3K activity in TCR signaling, further studies are required to address the physiological significance of SHP-1 effects on PI3K. It also remains to be determined whether SHP-1 effects on PI3K signaling *in vivo* reflect direct modulation of PI3K activity by SHP-1 and/or the capacity of SHP-1 to influence other PI3K modulatory signaling effectors by virtue of its interaction with PI3K. Investigation of these various possibilities represents a promising avenue to further elucidating the mechanisms whereby both SHP-1 and PI3K impact upon the signaling cascades linking TCR stimulation to cell response.

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